SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 33, No. 5, pp. 1163–1169, 2014

Printed in the USA

INTERSEX (TESTICULAR OOCYTES) IN LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) ON THE DELMARVA PENINSULA, USA

LANCE T. YONKOS,*† ELIZABETH A. FRIEDEL,‡ and DANIEL J. FISHER†‡
†Department of Environmental Science and Technology, University of Maryland, College Park, Maryland, USA
‡Wye Research and Education Center, University of Maryland, Queenstown, Maryland, USA

(Submitted 5 August 2013; Returned for Revision 3 September 2013; Accepted 29 January 2014)

Abstract: The authors describe the prevalence and severity of intersex in the form of testicular oocytes in largemouth bass (*Micropterus salmoides*) collected over a 5-yr period from a variety of surface waters on the Delmarva Peninsula, USA, a region dominated by poultry production and agricultural land use. During a survey from 2005 to 2007 of approximately 200 male specimens representing 6 fish and 2 frog species collected from numerous small-order streams on Delmarva, intersex was observed in only largemouth bass (system-wide prevalence 17%). During 2008 and 2009, testicular oocytes were encountered in male largemouth bass from 6 lakes and 1 large river system, with prevalence ranging from 33% to 88% (weighted arithmetic mean, 57%). The prevalence of testicular oocytes in largemouth bass from Delmarva lakes was comparable to the highest levels reported in a national US Geological Survey reconnaissance of this species, which also occurred in regions of the Atlantic coastal plain with intensive row-crop and animal agriculture. To the authors' knowledge, the present study represents the first report in the peer-reviewed scientific literature of testicular oocytes in fish on the Delmarva Peninsula. *Environ Toxicol Chem* 2014;33:1163–1169. © 2014 SETAC

Keywords: Histopathy Fish indices Endocrine disruptors *Micropterus salmoides* Delmarva Penninsula Testicular oocytes

INTRODUCTION

The occurrence of intersex in wild fish populations has received considerable attention in recent decades in both the scientific literature and the public press. The first widespread occurrence was reported in roach (*Rutilus rutilus*) within riverine systems in the United Kingdom [1]. Intersex has since been observed in fish species on all continents except Antarctica [2–4]. While variety exists, the most commonly reported form of intersex in fish is the presence of female germ cells, or oocytes, within the male gonad, a condition referred to as "testicular oocytes" [5]. The presence of testicular oocytes in normally gonochoristic species (i.e., species in which the phenotypic gender does not change during the adult life of the animal) is considered a pathological condition [6], and in such species it has been used as an indicator of exogenous exposure to estrogenic endocrine active compounds [1,7,8]. Higher prevalences of intersex roach, for example, have generally been observed downstream of major wastewater-treatment plants in English rivers compared with upstream populations [1].

Analysis of gonads collected between 1995 and 2004 from 16 freshwater fish species within 9 US river basins found intersex specimens at 34 of 111 sites sampled [9]. Testicular oocytes were the most prevalent form of intersex, with occurrence almost exclusively in smallmouth bass (*Micropterus dolomieu*) and largemouth bass (*Micropterus salmoides*). For each species, 44% of sites sampled possessed intersex fish, with an overall prevalence of 33% for smallmouth bass and 18% for largemouth bass. However, intersex largemouth bass were found at all 9 Atlantic coastal plain sites sampled (i.e., Apalachicola, Pee Dee, and Savannah River basins) with a mean prevalence of 66% (range, 30–91%). This region is dominated by row-crop production and intensive animal agriculture (predominantly poultry and swine), leading Hinck et al. [9] to predict that a high prevalence of testicular oocytes would likely also occur in largemouth bass from other regions with similar land-use characteristics.

The impetus for the present study was to investigate possible endocrine disruptive effects of poultry litter on aquatic biota within agriculturally impacted receiving waters. The selected research area, the Delmarva Peninsula (Figure 1), has an enormous poultry production industry (e.g., >600 million birds annually) on a proportionally small land area, resulting in one of the densest animal agriculture regions in the United States [10]. Likewise, poultry litter—the accumulated excreta, absorbent bedding, feathers, and uneaten food from commercial poultry houses—is predominantly land-applied as organic fertilizer for regionally intensive row-crop production [11]. Studies in our laboratory and elsewhere have shown that poultry litter contains abundant fecal estrogens (e.g., 17β-estradiol and estrone) that transport readily from agricultural fields to receiving waters via surface runoff and that retain sufficient estrogenicity to induce endocrine disruption in resident biota [12–14]. Because poultry production is the dominant industry on Delmarva and the vast majority of poultry waste is land-applied as fertilizer, an effort was undertaken to investigate the occurrence of intersex in aquatic biota resident within regional receiving waters. We present results from 5 yr of sampling within Delmarva inland waters including a survey of fish and frog species from numerous small-order streams and subsequent targeting of male largemouth bass from regional ponds, lakes, and rivers.

MATERIALS AND METHODS

Sampling sites

A preliminary stream survey was conducted between 2005 and 2007 to evaluate testicular oocyte prevalence across fish

All Supplemental Data may be found in the online version of this article.
* Address correspondence to lyonkos@umd.edu.
Published online 1 February 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.2544

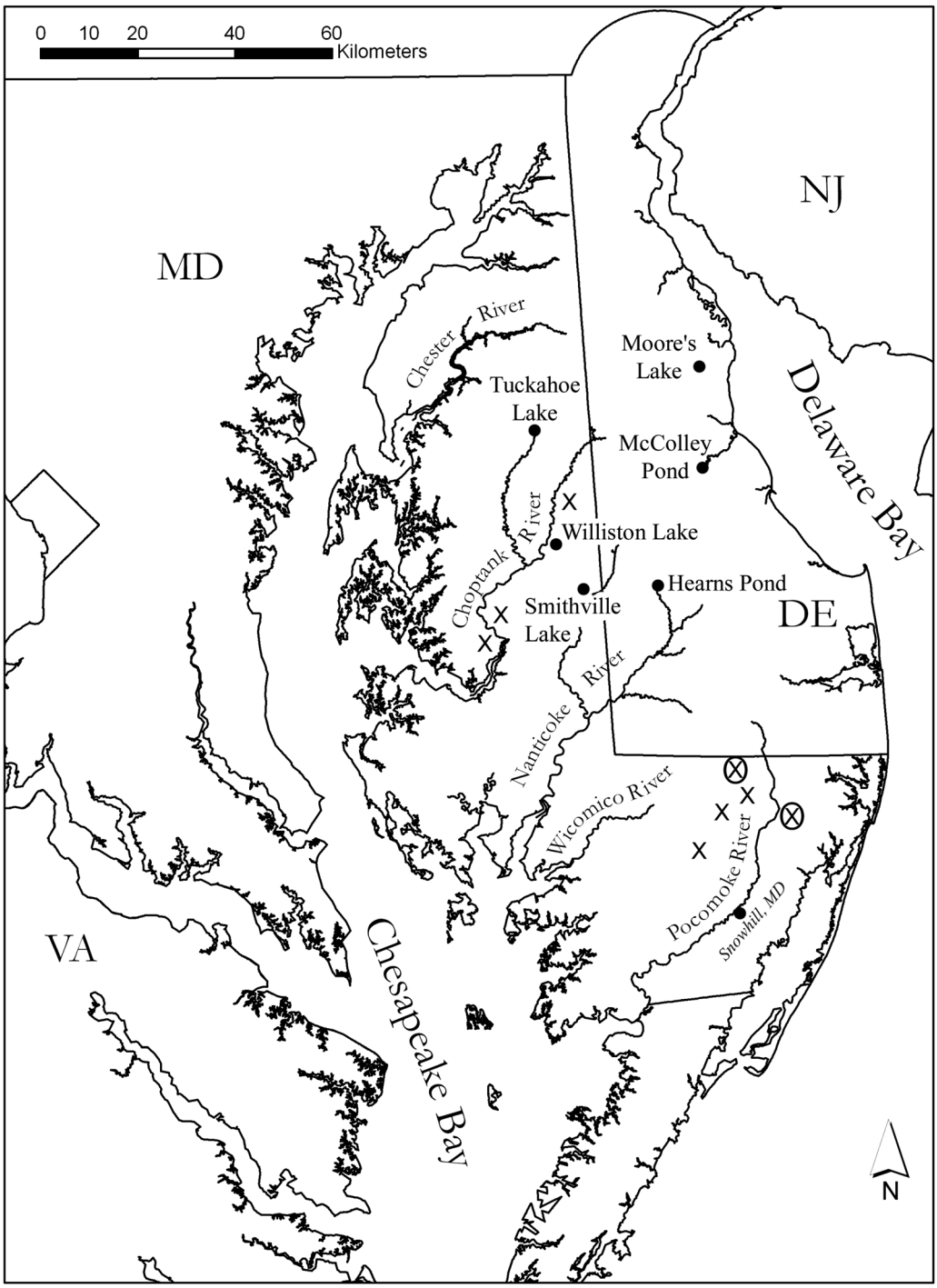

Figure 1. The Delmarva Peninsula, USA, with sites where mature male largemouth bass (*Micropterus salmoides*) were collected during the 2005 to 2007 small stream survey (×) and the 2008 to 2009 lake and river sampling (•). All lake and river sites had bass with testicular oocytes. Stream sites where testicular oocytes were observed (1 fish from each) are circled.

and frog species within tributaries of the Choptank and Pocomoke River systems (Figure 1). Selected survey sites ($n = 16$) were generally first- or second-order streams in regions of intense poultry production, with catchments dominated by agricultural land use. Criteria for site selection included adequate access for organism collection, sufficient year-round water volume for maintenance of aquatic habitat, and previous detection of estrogens (estrone and 17β-estradiol) in surface waters during and after 2004 spring rain events (L.T. Yonkos, unpublished data). Many sites were forested and occupied natural streambeds, whereas others were cleared of woody vegetation and channelized to facilitate agricultural field drainage. All sites received some proportion of input as runoff from agricultural fields, with many originating entirely as anastomosing field-side ditches. Collections occurred between August and October 2005, between June and July 2006, and during July 2007. Several sites were visited only in 2005, whereas others were revisited during 1 or both subsequent years.

During spring 2008, 6 lakes or ponds, all constructed via impoundment of small rivers or creeks, were visited: 3 each in the Maryland and Delaware portions of the peninsula (Figure 1). Sample sites within the Chesapeake Bay watershed included

Tuckahoe and Williston Lakes in the Choptank River basin and Smithville Lake and Hearns Pond in the Nanticoke River basin. The remaining sites, Moore's Lake and McColley Pond, drain to tidal estuaries associated with the Delaware Bay. Lakes were selected based on sufficient abundance of largemouth bass to ensure adequate capture of mature males without an adverse effect on populations. Lake catchments were delineated using digital US Geological Survey quarter quad topographic maps, and catchment areas were calculated in ArcMap 10.0.3 (Table 1) [15]. Land cover (percent agricultural, urban/suburban, and forest) was calculated for all lake catchments using the 2006 National Land Cover Database [16]. Mature male largemouth bass were collected again from Tuckahoe Lake in 2009 and from a 3-km segment of the Pocomoke River immediately above Snowhill, Maryland, USA (Figure 1). Collection in 2009 occurred once during spring (prespawn) and again during midsummer (postspawn) to investigate seasonal effects on testicular oocyte prevalence.

Specimen collection and processing

Fish and frog species were collected with permission from the Maryland Department of Natural Resources and the Delaware Department of Natural Resources and Environmental Control. Collection and subsequent processing of tissues for analysis were performed using protocols approved by the University of Maryland Institutional Animal Care and Use Committee. During the 2005 to 2007 survey, stream segments of 100 m to 300 m were sampled, with all fish $\geq$100 mm total length and all frogs $\geq$50 mm snout to vent length returned to the laboratory in aerated live wells and sacrificed for tissue collection (Supplemental Data, Table S1, reports those species for which a minimum of 8 mature males were collected to assess testicular oocyte occurrence). Collection was primarily via backpack electroshocker and occasionally via gill net. During 2008 and 2009, mature male largemouth bass were exclusively targeted for capture from lake and river sites via boat electrofishing. Fish were processed on location at a mobile field station to minimize handling stress and travel time before sacrifice and tissue preservation. Care was taken to select only largemouth bass $\geq$250 mm total length, a size at which males were presumed to be reproductively mature [17]. Urogenital opening characteristics were assessed and, where possible, gametes expressed to preferentially retain males, with obvious females released immediately [18]. On return to the field processing station, fish were anesthetized and gender was determined on-site to further minimize the unnecessary sacrifice of females. Collection was discontinued at each site once 10 males had been captured. In total, 94 male largemouth bass were collected from the various sites during 2008 and 2009 (Table 2).

All species of fish were anesthetized in 100 mg/L buffered tricaine methanesulfonate (Finquel; Argent Laboratories), observed for gross lesions, measured for total length to the

Table 1. Lake and catchment area and land-use characteristics within catchments of sampled lakes[a]

			Catchment land use (%)[b]		
Site (USA)	Lake area (hectares)	Catchment area (hectares)	Urban	Agriculture	Forest
Hearns Pond, DE	24.6	3484.4	10.8	71.0	7.3
Moore's Lake, DE	10.7	3544.4	22.9	59.5	8.3
McColley Pond, DE	25.0	4789.5	15.0	58.0	16.0
Williston Lake, MD	22.2	2572.0	5.2	63.1	15.9
Smithville Lake, MD	17.6	2515.4	6.0	57.3	16.4
Tuckahoe Lake, MD	24.9	22 434.6	4.3	64.8	13.3

[a]Catchments delineated using digital US Geological Survey quarter quad topographic maps with catchment area calculated using ArcMap 10.0.3 [15].
[b]Watershed area and land-use delineation determined using the 2006 data set of the National Land Cover Database [16]; sum of land use from given categories does not equal 100%; other land-use types (e.g., woody wetlands, open water) account for the remainder.

Table 2. Morphometric characteristics and testicular oocyte prevalence and severity in male largemouth bass (*Micropterus salmoides*) collected on the Delmarva Peninsula in 2008 and 2009[a]

						Testicular oocyte	
Site (USA)	Date collected	*n*	Length[b] (cm)	Weight[b] (g)	Condition factor[b,c]	Prevalence	Severity index
2008							
Hearns Pond, DE	30 April	8	36.9 ± 3.24	686 ± 160.7	1.34 ± 0.050	88%	0.22 ± 0.16
Moore's Lake, DE	30 April	10	35.1 ± 4.03	639 ± 220.7	1.43 ± 0.104	80%	0.37 ± 0.38
McColley Pond, DE	01 May	9	34.2 ± 5.40	588 ± 406.7	1.33 ± 0.177	67%	0.24 ± 0.26
Williston Lake, MD	15 May	11	38.4 ± 3.58	769 ± 223.4	1.34 ± 0.141	73%	0.26 ± 0.27
Smithville Lake, MD	22 May	10	36.1 ± 3.53	654 ± 163.0	1.37 ± 0.079	40%	0.11 ± 0.17
Tuckahoe Lake, MD	29 May	12	33.9 ± 5.18	572 ± 375.6	1.33 ± 0.166	42%	0.24 ± 0.47
2009							
Tuckahoe Lake, MD	12 May	10	36.6 ± 3.05 AB	730 ± 209.5	1.46 ± 0.145	50%	0.16 ± 0.21
Tuckahoe Lake, MD	19 August	9	37.4 ± 5.45 A	810 ± 406.1	1.41 ± 0.149	33%	0.25 ± 0.42
Pocomoke River, MD	14 May	10	34.9 ± 4.56 AB	629 ± 254.0	1.39 ± 0.166	40%	0.33 ± 0.56
Pocomoke River, MD	20 August	5[d]	30.1 ± 4.07 B	387 ± 193.2	1.32 ± 0.110	80%	0.53 ± 0.41

[a]Values followed by the same uppercase letter do not differ significantly from one another (Tukey's test, $p \leq 0.05$); absence of letters indicates no significant differences detected between groups; comparisons performed independently on data from each year.
[b]Length, weight, condition factor, and severity indices presented as mean ± standard deviation.
[c]Condition factor calculated by the formula (body weight/total length^3) × 100.
[d]Fewer than desired minimum sample number of 8.

nearest millimeter, weighed to the nearest 0.1 g, and bled via incision of the caudal vein before being sacrificed via decapitation. Testes were removed from male fish and fixed in 10% neutral buffered formalin for a minimum of 48 h before submission for routine histological preparation. Small testes were fixed whole, while larger organs were parceled into anterior, middle, and posterior segments. At least 3 segments from each testis lobe representing anterior, middle, and posterior regions were dehydrated in alcohol, embedded in paraffin, cross-sectioned at 6 μm, mounted on glass slides, and stained with hematoxylin and eosin [19]. For each specimen, an attempt was made to examine at least 1 histological section from each of the 6 tissue segments via light microscopy for testicular oocyte occurrence and other pathology. Segments from larger specimens were also step-sectioned (~200 μm between sections), allowing examination of additional tissue. The diameter of testis cross sections varied substantially. For most mature fish, testis cross sections ranged between 4 mm and 10 mm in diameter, the exception being larger largemouth bass collected during spring (prespawn), which often had testis cross sections of 15 mm to 20 mm. Therefore, the area of tissue available for examination often varied substantially between specimens.

Frogs were treated similarly to fish but required additional Finquel (generally ≥1.0 g/L) to achieve anesthesis. Following anesthesis, frog testes were removed, fixed (10% neutral buffered formalin), trimmed, and processed while paired and attached to the kidney to maintain tissue orientation. Smaller frog testes (≤5 mm in length) were blocked whole, whereas larger organs were divided into anterior and posterior segments before blocking. In each case, blocks were step-sectioned (~200 μm) to yield tissue sections from multiple regions of the organs.

Testicular oocyte quantification

Tabulation of testicular oocyte prevalence was based on observation of 1 or more discernible oocytes within preserved cross sections of testicular tissue from individual specimens. Briefly, all tissue sections of adequate quality from each specimen were scanned for the presence of oocytes under low and moderate magnification (4× and 10× objectives, respectively) with confirmation of presumptive oocytes made under high magnification (40× objective). For the small-order stream samples, all male fish and frogs with testes sufficiently developed to remove and process histologically were examined. Numbers of males varied widely between collected species, reflecting variations in population densities encountered at the sample sites and the proportion of mature males among those captured (Supplemental Data, Table S1).

Assessment of testicular oocyte severity in largemouth bass conformed as closely as possible to the ranking system described by Blazer et al. [20] for smallmouth bass. Briefly, the central regions of testis cross sections were examined under moderate magnification (10× objective), and testicular oocyte occurrence was ranked as follows: focal distribution (score 1), a single oocyte within a microscope field; diffuse distribution (score 2), more than 1 spatially distinct oocyte within a microscope field; cluster distribution (score 3), more than 1 but fewer than 5 closely associated oocytes within a microscope field; and zonal distribution (score 4), multiple oocyte clusters within a microscope field (see Blazer et al. [20] for detailed description). All histological sections for individual specimens were scored for testicular oocyte severity (number of sections per specimen ranged from as few as 2 to as many as 14), with the average of resulting scores used to establish the individual fish severity index. Where multiple fields of view for a given section were scored with differing results, the most severe rank was used. Site average severity indices were generated by calculating the mean of individual specimen scores.

Statistical analysis

Quantitative data (e.g., morphometric characteristics and testicular oocyte severity indices) were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Where data failed the assumptions of normality or homogeneity of variance, Kruskal-Wallis ANOVA on ranks was employed. Proportion data (e.g., testicular oocyte prevalence) were compared across sample sites using a chi-squared test and between seasons (e.g., prespawn vs postspawn) using a Fisher's exact test. All analyses were performed using SigmaStat version 3.5 (Systat Software), with statistical significance reported at $p = 0.05$.

RESULTS

Small-order stream sampling: 2005 to 2007

A total of 424 specimens from 8 species (creek chubsucker, *Erimyzon oblongus*; redfin pickerel, *Esox americanus*; white perch, *Morone americana*; yellow perch, *Perca flavescens*; largemouth bass, *Micropterus salmoides*; common carp, *Cyprinus carpio*; northern green frog, *Rana clamitans*; American bullfrog, *Rana catesbeiana*) were collected during the 3-yr stream survey (Supplemental Data, Table S1). Examination of testicular tissues from those specimens determined to be mature males ($n = 192$) revealed the presence of testicular oocytes only in largemouth bass (Figure 2). Prevalence and severity were low, with only 2 of 12 mature male fish having only occasional focally distributed oocytes (individual severity indices 0.4 and 0.6). The abundance of mature male largemouth bass collected from small-order streams was insufficient for meaningful calculation of site average testicular oocyte prevalence or severity. System-wide over the 3-yr stream survey testicular oocyte prevalence in largemouth bass was 17% and the severity index was approximately 0.05.

Lake and river sampling: 2008 to 2009

Land use within the catchments of all 6 Delmarva lakes sampled during 2008 was dominated by agriculture (range, 57–71%, Table 1), with most of the area dedicated to corn and soybean production necessary to satisfy feed requirements for the substantial regional poultry industry. The percentage of urban and suburban land use varied by >5-fold between sites, with the Moore's Lake catchment having the greatest amount of urban land use (22.9%) and Tuckahoe Lake having the least (4.3%). The remaining area was comprised of forest, woody wetlands, and open water. Sampling from the 6 lakes yielded 60 mature male largemouth bass (Table 2). Total length, weight, and condition factor did not differ significantly between lakes. Intersex was observed in fish from all 6 lakes (Table 2; Supplemental Data, Figure S1). Collectively, 38 individuals (63%) were found to possess testicular oocytes with lake-specific prevalence ranging from 40% to 88% and average severity indices ranging from 0.11 to 0.37. Severity was not found to differ significantly between lakes (Kruskal-Wallis one-way ANOVA, $p = 0.33$). Prevalence also did not differ between lakes (chi-squared, $p = 0.17$; because of the small sample size, the power of the test [0.54] was below the desired level of 0.80, suggesting that negative results be interpreted cautiously).

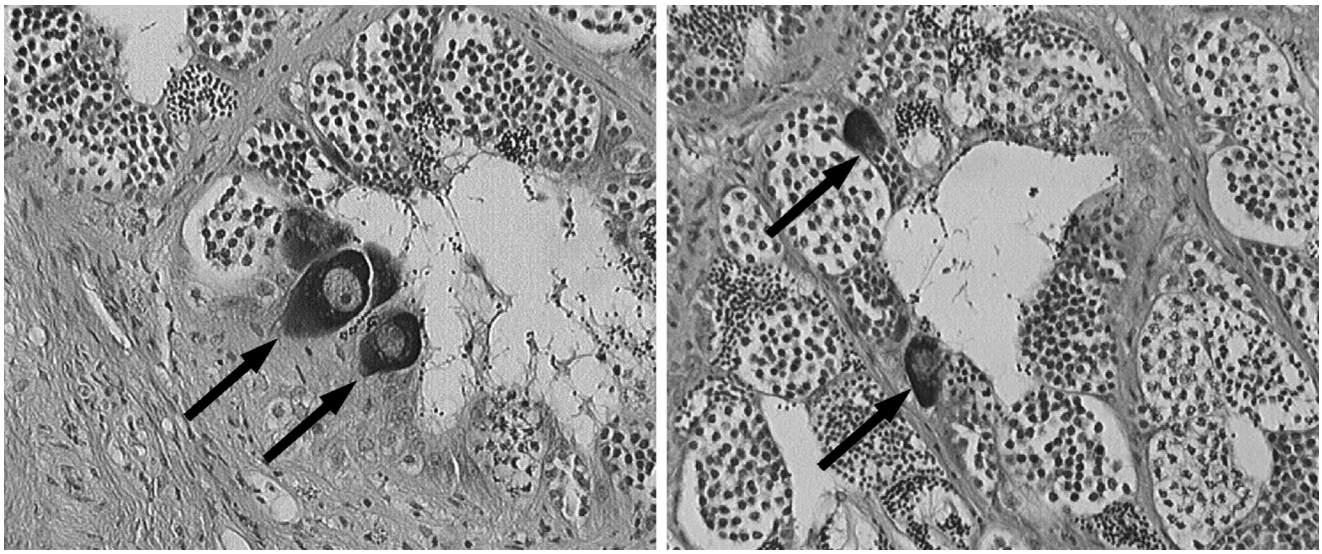

Figure 2. Testicular oocytes in largemouth bass (*Micropterus salmoides*) from the Delmarva Peninsula, USA. Clustered (**A**) and diffuse (**B**) previtellogenic oocytes (arrows) within the epithelium of testicular tubules (bar = 50 μm, hematoxylin and eosin stain).

In 2009, a total of 34 mature male largemouth bass were collected from Tuckahoe Lake and from the Pocomoke River during spring (prespawn) and summer (postspawn) sampling (Table 2). While only 5 fish were collected from the Pocomoke during summer sampling (below the desired sample number of 8), the authors feel that detection of oocytes in 4 of 5 male fish indicates testicular oocyte prevalence was moderate to high and justifies inclusion of the data. Statistical results, however, should be interpreted with caution in light of this comparatively small sample size. Weight and condition factor did not differ significantly between fish from the various sample locations and times. However, the mean length of fish collected during the summer (postspawn) from the Pocomoke River was less than that of postspawn fish from Tuckahoe Lake (one-way ANOVA followed by Tukey's test, $p \leq 0.05$; Table 2). Prevalence of testicular oocytes in Tuckahoe Lake fish in 2009 was 50% and 33% during spring and summer sampling, respectively (Table 2). Prevalence in Pocomoke River fish was 40% and 80% during spring and summer sampling, respectively. Site average severity indices for the 2 systems ranged from 0.16 to 0.53. Despite the wide range, severity indices did not differ significantly between sample sites and dates (Kruskal-Wallis one-way ANOVA, $p = 0.40$). Likewise, testicular oocyte prevalence was not found to differ between prespawn and postspawn fish from Tuckahoe Lake (Fisher's exact test, $p = 0.65$) or the Pocomoke River (Fisher's exact test, $p = 0.28$).

DISCUSSION

In their survey of 16 fish species within 9 US river basins, Hinck et al. [9] found only largemouth bass and smallmouth bass to have any appreciable occurrence of intersex and only in the form of males with testicular oocytes. The retrospective study (1995–2004), which examined gonads from 3110 fish, found that only 97 specimens were intersexed (3.1%), with 93 of those being male black bass (i.e., smallmouth bass and largemouth bass) possessing testicular oocytes. The results of the present small-order stream survey are similar. In examination of 192 male specimens representing many of the larger fish and frog species commonly found in Delmarva inland waters, only largemouth bass were found to possess testicular oocytes; smallmouth bass are not present on Delmarva. Intersex in the form of testicular oocytes has been reported for several of the other fish and frog species we examined. White perch (*Morone americana*) from industrial regions on the Great Lakes, USA, had testicular oocytes levels of 22% to 83% [21]. Similarly, common carp (*Cyprinus carpio*) have been reported with testicular oocytes in several industrialized European rivers [22,23]. In the present study, male white perch were collected in adequate numbers ($n = 16$) to assess testicular oocyte prevalence. Lack of detection suggests that testicular oocytes either are absent or occur only at a low prevalence in this species. In contrast, only 8 male common carp and yellow perch were collected. While testicular oocytes were not observed in these species, this is an inadequate sample size to make any strong statement about intersex prevalence. Intersex has also been reported in the northern green frog (*Rana clamitans*), a species we collected in abundance. Skelly et al. [24] found testicular oocytes in 13% of male northern green frogs collected from ponds within the Connecticut River watershed. Prevalence was highest in urban and suburban landscapes (16%–21%), moderate in agricultural landscapes (5%), and nonexistent in forested/undeveloped landscapes (0%). None of the 60 male northern green frogs we collected from numerous small-order Delmarva streams was found to possess testicular oocytes. Our collection from lotic (e.g., stream) versus lentic (e.g., pond) systems may explain this difference.

Collectively, in our 2008 to 2009 lake and river sampling, 54 of 94 male largemouth bass (57%) were found to possess testicular oocytes, with site-specific prevalence ranging from 33% to 88%. To the best of our knowledge, the present study represents the first description in the peer-reviewed scientific literature of intersex in aquatic biota from the Delmarva Peninsula (testicular oocytes were previously observed in largemouth bass collected from Tuckahoe Lake in 2006 by M. Matsche, Maryland Department of Natural Resources, Annapolis, Maryland, USA, personal communication). Prevalence of testicular oocytes in lake-collected Delmarva largemouth bass was comparable to the highest levels reported elsewhere for this species [9,25] and considerably higher than the sole report in the scientific literature of testicular oocytes in largemouth bass from the Chesapeake Bay region, a 23%

prevalence (2 of 9) in fish collected near the discharge of a large wastewater-treatment plant in the Potomac River [26]. Intersex prevalence in lake-collected Delmarva largemouth bass was similar to levels observed in smallmouth bass in other regions of the Chesapeake Bay watershed [8,26] and elsewhere in the United States [9,25,27]. Testicular oocytes were reported in smallmouth bass from various reaches of the Potomac River system in 2003 to 2005 [20] and again in 2006 to 2007 [8,26]. Prevalence in smallmouth bass across Potomac River sites over this 5-yr period ranged from 25% to 100%, similar to the 33% to 88% prevalence range we found in largemouth bass from Delmarva lakes and the Pocomoke River. Site-average severity indices were generally lower in Delmarva largemouth bass (e.g., 0.1–0.5) than those reported in smallmouth bass from the Potomac River system (e.g., 0.3–1.8) but higher than those reported for smallmouth bass from reference sites outside the Potomac drainage (e.g., 0.02–0.2) [8,20]. The range of site-average severity index in Delmarva largemouth bass bracketed the lone reported severity index of 0.2 in Potomac River largemouth bass [26], suggesting comparable testicular oocyte severity in largemouth bass from the 2 systems.

For a number of reasons, specific comparison between studies should be undertaken with caution. Histological detection of testicular oocytes within an individual fish depends on severity (i.e., the abundance of oocytes within the testis) but also on the method of tissue preservation and the amount, location, and orientation of tissue examined [20]. In the case of black bass (*Micropterus* spp.), sections should be transverse and pass through the central region of the testis (where oocytes occur most frequently) to ensure a reasonable likelihood of encountering testicular oocytes. As demonstrated by Blazer et al. [20], if a fish's testicular oocyte severity index exceeds 0.5, then analysis of 5 appropriate cross sections yields a 90% probability of detection. However, where severity is low (e.g., severity index $\leq$0.2), as many as 10 sections are necessary to achieve a similar 90% detection probability. The number of sections or specimens examined for the current Delmarva study varied from 2 to 14 but was generally around 6. Resulting site-average severity indices for largemouth bass collected in 2008 and 2009 were $<$0.5 for all locations but 1. Since the number of sections examined for any given fish influences the probability of testicular oocyte detection for that fish, testicular oocyte prevalence is more accurately reflected in cases where high numbers of sections were examined and perhaps underestimated for those cases where relatively few sections were available. Similar discrepancies in methodology are common. In the most comprehensive survey to date of intersex in US rivers, Hinck et al. [9] reported that "two to eight pieces of gonad were collected" and "samples were generally transverse sections" but "whole gonad was embedded and sectioned longitudinally if the gonad was very small." Differences in number, orientation, and location of sections examined likely had some influence on oocytes detected and thus on reported testicular oocyte prevalence. In a study of smallmouth bass from the polychlorinated biphenyl (PCB)–contaminated Kalamazoo River, Michigan, USA, Anderson et al. [27] described collecting transverse slices of testis from cranial, middle, and caudal thirds and assessing testicular oocytes using a system of their own devising to estimate severity. Their ranking system tabulates number of oocytes within a single microscopic field under 20$\times$ objective (significantly less observed area than under the 10$\times$ objective used by Blazer et al. [20] and in the present study). This notwithstanding, the researchers report 100% testicular oocyte prevalence, suggesting either very high severity or that numerous sections were examined. Baldigo et al. [25], however, describe subsamples of gonads being fixed and submitted for histological analysis but provide no information on the number of subsamples or specimens, the location(s) from which subsamples were taken, or the ultimate number of histological sections examined. Without this information, reported smallmouth bass and largemouth bass testicular oocyte prevalences (at sites where detected) of 20% to 50% may not be explicitly comparable to numerically similar results from other studies.

Hinck et al. [9] found a high prevalence of intersex black bass at sites with dense human populations (i.e., substantial wastewater-treatment plant influence) but also in regions of intense agricultural activity (2 sites with high testicular oocyte prevalence were in areas where agriculture was the exclusive land use). The authors found no consistent relationship between prevalence of intersex and body burdens of any of 24 measured bioaccumulative contaminants (e.g., organochlorine pesticides, PCBs, tetrachlorodibenzo-*p*-dioxins, and total Hg). The authors did note, however, in relation to their discovery of intersex largemouth bass at all sites sampled from the Pee Dee, Savannah, and Apalachicola River basins, that testicular oocytes might be unusually prevalent in regions where row-crops and animal agriculture dominate land use. The Delmarva Peninsula is just such a region. Poultry production is the dominant industry, with feed requirements satisfied by intensive regional corn and soybean cultivation and disposal of poultry waste principally via agricultural application as organic fertilizer [28]. All lakes sampled on Delmarva had high levels of agricultural activity within catchments and, as predicted by Hinck et al. [9], high testicular oocyte prevalence. In the present study the degree of similarity in agricultural land use between sites (range, 57–71%) precludes meaningful investigation into correlations between percentage of agriculture and testicular oocyte prevalence or severity. An apparent lack of association between percentage of urban and suburban development and testicular oocyte prevalence is potentially more meaningful (e.g., the highest testicular oocyte prevalence [88%] occurred in Hearns Pond, a site with only moderate urban land use [11%]). It is worth noting that Moore's Lake, the site with the highest urban land use at 22.9%, had a testicular oocyte prevalence of 80%. This site has also been under a finfish consumption advisory since 1999, aimed at limiting recreational anglers' exposure to PCBs and DDT [29]. Because sample sites for the present study were relatively few in number and had significant similarities in land use, no definitive statement can be made concerning relationships between intersex prevalence and severity and various land-use characteristics. To address this shortcoming, future investigations on Delmarva should include sites with varying degrees of agricultural and urban land use and reference sites with minimal development.

SUPPLEMENTAL DATA

Table S1.
Figure S1. (143 KB PDF).

Acknowledgment—The present research was supported by the US Environmental Protection Agency (STAR Grant EPA-G2006-STAR-M1) and the US Department of Agriculture via the Harry R. Hughes Center for Agro-Ecology (Queenstown, MD). We thank J. Kilian, S. Stranko, A. Prochaska, and P. Ciccotto (Maryland Department of Natural Resources) and C. Martin (Delaware Department of Natural Resource and Environmental Control) for fish collections; A. Becker and S. McNally (Maryland Department of Natural Resources) for watershed land-use delineations; and L. Hamilton and J. Blazek (Maryland Department of Natural Resources) for histological preparations.

REFERENCES

1. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter J. 1998. Widespread sexual disruption in wild fish. *Environ Sci Technol* 31:2498–2506.
2. Hashimoto S, Bessho H, Hara A, Nakamura M, Iguchi T, Fujita K. 2000. Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. *Mar Environ Res* 49:37–45.
3. Barnhoorn IE, Bornman MS, Pieterse GM, Van Vuren JH. 2004. Histological evidence of intersex in feral sharptooth catfish (*Clarias gariepinus*) from an estrogen-polluted water source in Gauteng, South Africa. *Environ Toxicol* 19:603–608.
4. de Sá MFP, Fragoso-Moura EN, Fenerich-Verani N, Ferro DAM. 2008. Occurrence of intersexuality in "Lambaris," *Astyanax scabripinnis* (Jenyns, 1842), small characids from the Brazilian streams. *Braz Arch Biol Technol* 51:315–322.
5. Nolan M, Jobling S, Brighty G, Sumpter JP, Tyler CR. 2001. A histological description of intersexuality in the roach. *J Fish Biol* 58:160–176.
6. Hecker M, Murphy MB, Coady KK, Villeneuve DL, Jones PD, Carr JA, Solomon KR, Smith EE, Van Der Kraak G, Gross T, Du Preez L, Kendall RJ, Giesy JP. 2006. Terminology of gonadal anomalies in fish and amphibians resulting from chemical exposures. *Rev Environ Contam Toxicol* 187:103–131.
7. Allen Y, Mattheissen P, Scott AP, Howarth S, Feist S, Thain JE. 1999. The extent of oestrogenic contamination in the UK estuarine and marine environments—Further studies of flounder. *Sci Total Environ* 233:5–50.
8. Blazer VS, Iwanowicz LR, Henderson H, Mazik PM, Jenkins JA, Alverez DA, Young JA. 2012. Reproductive endocrine disruption in smallmouth bass (*Micropterus dolomieu*) in the Potomac River basin: Spatial and temporal comparisons of biological effects. *Environ Monit Assess* 184:4309–4334.
9. Hinck JE, Blazer VS, Schmitt CJ, Papoulias DM, Tillitt DE. 2009. Widespread occurrence of intersex in black basses (*Micropterus* spp.) from U.S. rivers, 1995-2004. *Aquat Toxicol* 95:60–70.
10. US Department of Agriculture. 2009. *2007 Census of Agriculture*. National Agricultural Statistics Service, Washington, DC.
11. Moore PA, Daniel TC, Edwards DR, Miller DM. 1996. Evaluation of chemical amendments to reduce ammonia volatilization from poultry litter. *Poult Sci* 75:315–320.
12. Shore LS, Correll DI, Chakraborty PK. 1995. Relationship of fertilization with chicken manure and concentrations of estrogens in small streams. In Steele K, ed, *Animal Waste and the Land–Water Interface*. CRC Press, Boca Raton, FL, USA, pp 155–163.
13. Nichols DJ, Daniel TC, Edwards DR, Moore PA Jr, Pote DH. 1998. Use of grass filter strips to reduce 17β-estradiol in runoff from fescue-applied poultry litter. *J Soil Water Conserv* 53:74–77.
14. Yonkos LT, Fisher DJ, Van Veld PA, Kane AS, McGee BL, Staver KW. 2010. Poultry litter–induced endocrine disruption in fathead minnow, sheepshead minnow and mummichog laboratory exposures. *Environ Toxicol Chem* 29:2328–2340.
15. Environmental Systems Resource Institute. 2013. ArcMap 10.0.3. Redlands, CA, USA.
16. Fry J, Xian G, Jin S, Dewitz J, Homer C, Yang L, Barnes C, Herold N, Wickham J. 2011. Completion of the 2006 National Land Cover Database for the conterminous United States. *Photogrammetric Engineering and Remote Sensing* 77:858–864.
17. Davis JT, Lock JT. 1997. Largemouth bass biology and life history. SRAC Publication No. 200. Southern Regional Aquaculture Center, US Department of Agriculture, Cooperative States Research, Education, and Extension Service. Stoneville, MS.
18. Parker WD. 1971. Preliminary studies on sexing adult largemouth by means of an external characteristic. *Progressive Fish Culturist* 36: 55–56.
19. Luna LG. 1992. *Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts*. American Histolabs, Gaithersburg, MD, USA.
20. Blazer VS, Iwanowicz LR, Iwanowicz DD, Smith DR, Young JA, Hedrick JD, Foster SW, Reeser SJ. 2007. Intersex (testicular oocytes) in smallmouth bass *Micropterus dolomieu* from the Potomac River and selected nearby drainages. *J Aquat Anim Health* 19:242–253.
21. Kavanagh RJ, Balch GC, Kiparissis Y, Niimi AJ, Sherry J, Tinson C, Metcalfe CD. 2004. Endocrine disruption and altered gonadal development in white perch (*Morone americana*) from the lower Great Lakes region. *Environ Health Perspect* 112:898–902.
22. Solé M, Raldua D, Pifferrer F, Barceló D, Porte C. 2003. Feminization of wild carp, *Cyprinus carpio*, in a polluted environment: Plasma steroid hormones, gonadal morphology and xenobiotic metabolizing system. *Comp Biochem Physiol C* 136:145–156.
23. Ramon L, Thibaut R, Raldúa D, Martín R, Porte C. 2004. First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicol Appl Pharmacol* 196:247–257.
24. Skelly DK, Bolden SR, Dion KB. 2010. Intersex frogs concentrated in suburban and urban landscapes. *EcoHealth* 7:374–379.
25. Baldigo BP, Sloan RJ, Smith SB, Denslow ND, Blazer VS, Gross TS. 2006. Polychlorinated biphenyls, mercury, and potential endocrine disruption in fish from the Hudson River, New York, USA. *Aquatic Science* 68:206–228.
26. Iwanowicz LR, Blazer VS, Guy CP, Pinkney AE, Mullican JE, Alvarez DA. 2009. Reproductive health of bass in the Potomac, USA drainage: Part 1. Exploring the effects of proximity to wastewater treatment plant discharge. *Environ Toxicol Chem* 28:1072–1083.
27. Anderson MJ, Cacela D, Beltman D, Teh SJ, Okihiro MS, Hinton DE, Denslow N, Zelikoff JT. 2003. Biochemical and toxicopathic biomarkers assessed in smallmouth bass recovered from a polychlorinated biphenyl–contaminated river. *Biomarkers* 8:371–393.
28. Staver KW, Brinsfield RB. 2001. Agriculture and water quality on the Maryland eastern shore: Where do we go from here? *Bioscience* 51:859–868.
29. Delaware Department of Natural Resources and Environmental Control. 2010-2011. [cited 2014 January 21]. Available from: http://www.dnrec.delaware.gov/fw/Fisheries/Documents/Delaware_Fish_Advisory_Chart.pdf.